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The Laboratory Culture of Two Aplysiids, *Aplysia brasiliana* Rang, 1828, and *Bursatella leachii plei* (Rang, 1828) (Gastropoda: Opisthobranchia) in Artificial Seawater

by

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Abstract. Techniques for the laboratory culture of the sea hares *Aplysia brasiliana* Rang, 1828, and *Bursatella leachii plei* (Rang, 1828) in artificial seawater are described. The simplified methods allowed high yields (70–80% of the starting density) to metamorphic competency. The larvae of both species grew an average of 10 μm per day and were successfully metamorphosed in artificial seawater on appropriate substrata—red algae for *A. brasiliana* and blue-green algae for *B. l. plei*. These methods provide a basis for investigations into the chemical requirements of larval development in a defined medium.

INTRODUCTION

SEA HARES (Opisthobranchia: Anaspidea) have been the focal point for an extensive amount of biomedical research during the past two decades. They have played an important role in our understanding of the cellular and molecular aspects of nervous function and the control of behavior (KANDEL, 1976, 1979). With the introduction of laboratory techniques for the culture of sea hares (KRIEGSTEIN *et al.*, 1974; STRENTH & BLANKENSHIP, 1978; SWITZER-DUNLAP & HADFIELD, 1977, 1981), research interest in these forms has expanded to include the development of neural systems and behavior (JACOB, 1984; KANDEL *et al.*, 1980; KRIEGSTEIN, 1977a; SCHACHER *et al.*, 1979a, b).

The major bottleneck in the culture of aplysiids is the planktonic larval phase, which often requires 30–35 days to complete. Previous methods have utilized natural seawater as the culture medium. At inland laboratories, where access to natural seawater is limited, and at coastal laboratories, where natural seawater quality is variable or unfit for culture, research on the development of these forms is difficult. The use of a defined medium would provide standardized environmental conditions throughout the life cycle and provide a basis for research into the chemical requirements for optimum development and physiological function. This paper is a report on the successful culture of two aplysiid species in artificial seawater.

MATERIALS AND METHODS

Maintenance of Breeders and Eggs

Mature adults of *Aplysia brasiliana* Rang, 1828, and *Bursatella leachii plei* (Rang, 1828) were collected from shallow-water habitats around Florida. These breeders were maintained in laboratory aquaria in Instant Ocean Sea Salts (ASW) at 32–35 ppt and 20–24°C. Daily they were fed freshly collected or laboratory cultured algae. Both species normally spawned large masses of eggs within a few days after capture and attached the egg strands to the aquarium walls or vegetation. The eggs were isolated from the adults by means of a perforated partition and were left where deposited to ensure that the environ-

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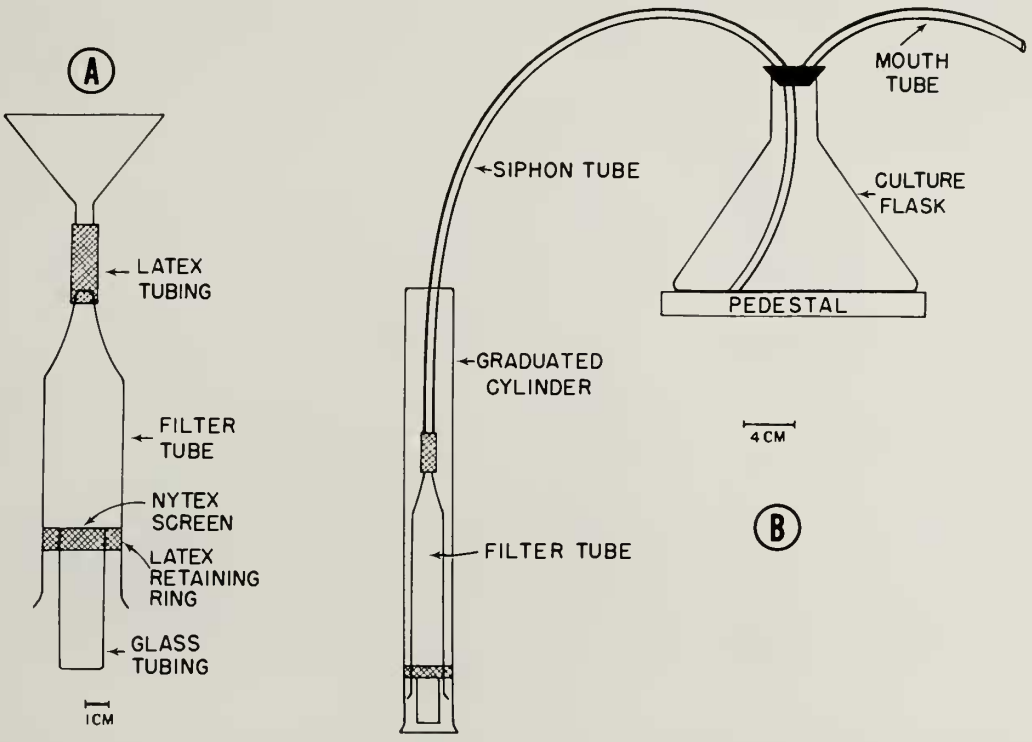

Figure 1

Apparatus for larval transfer. A. Filter unit. B. Larval culture and filter assembly.

mental conditions for embryonic development remained stable. Occasionally, eggs were removed and placed in a one-gallon jar in approximately 3.0 L of prefiltered ASW. These cultures were gently aerated and sealed with cellophane to prevent evaporation. Both techniques worked well.

Just prior to hatching (8–9 days after deposition), as evidenced by the brown coloration of the egg mass, small sections (1–2 cm) were cut from the center of the mass and placed in a bowl filled with prefiltered ASW, where they remained until hatching.

Larval Culture

Larval culture was carried out in a 3-L, low-form Erlenmeyer flask. This vessel had several advantages. The low, wide base allowed ample light penetration to enhance the growth of single-celled algae used as the larval food source. In addition, this flask had a narrow opening which minimized the area of surface where larvae could become trapped, a common problem in opisthobranch culture (Franz, 1975). The culture medium (ASW) was mixed to the desired salinity (33–35 ppt) in glass-distilled water. After the salts had dissolved, the solution was aerated for three days to ensure pH stability at 8.2–8.4. Prior to use in the larval cultures, the seawater was prefiltered to remove precipitates. No apparent benefit was derived from using one or a combination of the antibiotics Penicillin G, Streptomycin Sulfate, or Rifampicin, so no antibiotics were used.

After hatching, larvae were added to a flask to a final density of one larva per 10 mL of seawater. They were fed the single-celled alga *Isochrysis galbana* Parke, which was added directly from stock cultures grown in Guillard's "f/2" medium (Guillard, 1975) in ASW. The final concentration in the larval cultures was 10^4 algal cells per mL. The flask was filled to the top with ASW and sealed with Parafilm so as to eliminate completely air bubbles. These cultures were incubated at 24 ± 1°C under continuous illumination from one fluorescent light. Cultures could be maintained without transfer for up to 12 days. Routinely, they were transferred every 5–7 days using the siphoning apparatus shown in Figure 1.

The filter tube (Figure 1A) was constructed from glass tubing (22 mm I.D.). One end was pulled out to a narrow neck (5 mm I.D.) and the opposite end was flared to facilitate the insertion of the filter. The filter was made of glass tubing (18 mm O.D.) to which a small piece of nylon screen was attached by means of latex tubing (12 mm I.D., 18 mm O.D.). The filter was pushed up into the filter tube, producing a tight seal between the latex ring and the filter tube, to prevent larvae from passing

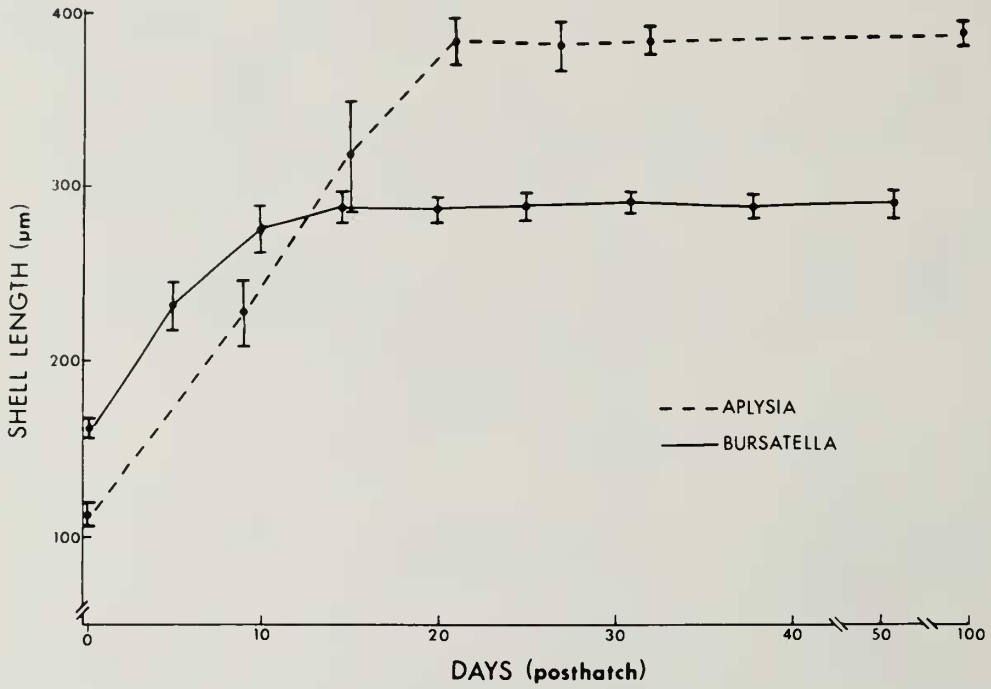

Figure 2

Larval growth of *Aplysia brasiliana* and *Bursatella leachii plei* in artificial culture. Each point represents the average shell size (maximum dimension) of 50 individuals. The bar is ±1 standard deviation.

through. Several of these units were made with screens of various mesh sizes (90–220 μm) to retain different sizes of larvae.

To transfer the larvae, the filter unit with the funnel attached (Figure 1A) was placed in a graduated cylinder that had been filled with prefiltered ASW. The larval culture vessel was opened and a small amount of seawater was poured into the funnel. A siphon tube was attached as shown in Figure 1B. By sucking on the mouth tube, air and water were pulled from the filter tube into the flask. Once all the air had been removed from the tubing, suction was stopped and an automatic siphon started that drained the flask, retaining the larvae on the screen. Water in the graduated cylinder was allowed to overflow into a gallon jar. This water was prefiltered and reused for the same culture, thus minimizing the need for excessive amounts of ASW. Routinely, culture water was reused 2 or 3 times and then discarded; however, recycled water could be used up to 70 days in culture before any detrimental effects were noted. After the flask was emptied, the funnel was reattached and all tubing and glassware were rinsed with fresh ASW to remove trapped larvae. The latex tubing was clamped and the funnel removed. The larvae could be dispensed directly into a new culture, or a bowl for observation, by inverting the filter unit and releasing the clamp. The screen was flushed with ASW to wash off remaining larvae. The larvae of both species were transferred and cultured in this manner until they showed signs of competency to metamorphose. Competency of larval *Aplysia brasiliana* was determined by the appearance of pigmented lateral spots and a mantle line according to KRIEGSTEIN (1977b). Because no lateral spots or mantle line appear in larval *Bursatella leachii plei,* competency was determined by the initiation of crawl-search behavior.

Larval Settlement and Postlarval Culture

Competent larvae were exposed to an appropriate settlement substrate using the techniques of SWITZER-DUNLAP & HADFIELD (1977). Ten to twenty larvae were placed in a small bowl in 100 mL of prefiltered ASW and were fed *Isochrysis galbana* at 10^4 cells per mL. Small pieces of substrate—red algae for *Aplysia brasiliana* and blue-green algae for *Bursatella leachii plei* (PAIGE, 1981)—were added. Cetyl alcohol was lightly sprinkled over the surface to prevent larval entrapment (HURST, 1967). The bowls were covered and incubated under continuous illumination at 22–24°C.

After settlement, the postlarvae were isolated in small bowls and fed the alga that had induced metamorphosis. Juveniles were maintained in this manner until they were large enough to be placed in aquaria.

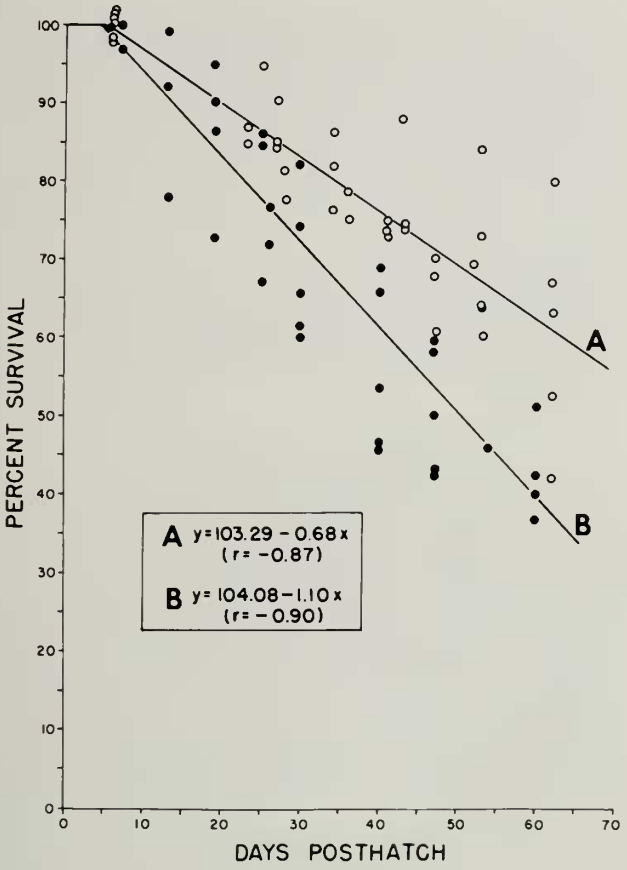

Figure 3

Percent survival of *Aplysia brasiliana* larvae in artificial seawater culture. Regression line A represents survivorship data (open circles) for six cultures of second generation larvae (n = 1846). Regression line B represents survivorship data (closed circles) for five cultures of first generation larvae (n = 1516).

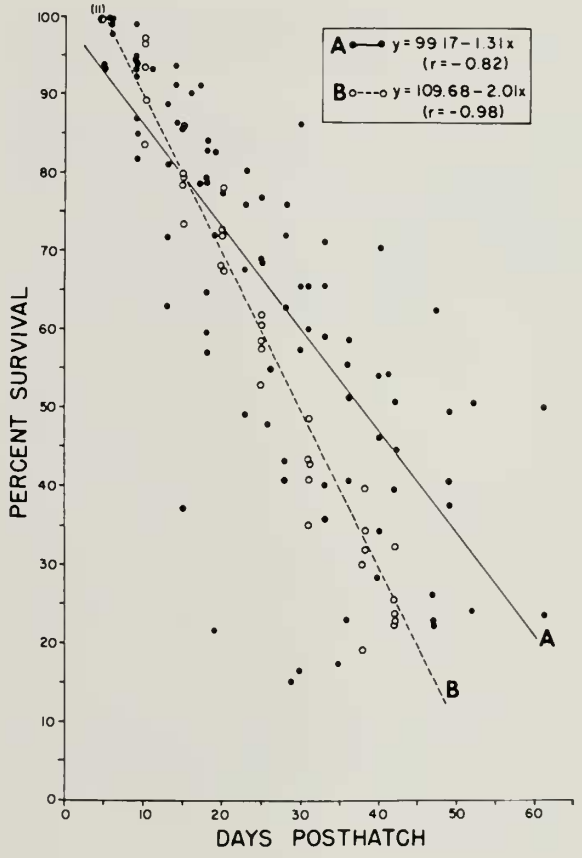

Figure 4

Percent survival of *Bursatella leachii plei* in artificial seawater culture. Regression line A represents survivorship data (closed circles) for thirteen cultures of larvae (n = 3988). Regression line B represents survivorship data (open circles) for five cultures of larvae (n = 1471).

RESULTS

The egg masses of *Aplysia brasiliana* and *Bursatella leachii plei* were characteristic of aplysiid spawn. The eggs were deposited in transparent capsules, embedded in a long, tangled gelatinous cordon. Each capsule contained from 1 to 30 eggs, depending on the size of the adult depositing the mass. The color of the egg mass varied, but was normally yellow to light green for both species. The color changed to dark brown near the end of embryonic development, which required 8–9 days to complete. Development and hatching in ASW was complete and no detrimental effects of the medium or disease were observed.

The shell size at hatching was species specific. The larvae of *Aplysia brasiliana* hatched at a maximum shell diameter of 111 ± 7 μm, whereas newly hatched *Bursatella leachii plei* larvae were 160 ± 4 μm. Larval growth in ASW (Figure 2) was approximately linear and averaged 10 μm per day for both species. The growth of *Aplysia brasiliana* veligers stopped in 21 days posthatch at a maximum shell size of 382 ± 14 μm. *Bursatella l. plei* larvae stopped growing in 15 days at a shell size of 286 ± 9 μm. At this time in development, the larvae of both species were not competent to metamorphose, even if exposed to the substrate on which they would eventually settle. *Aplysia brasiliana* larvae required 34 days to reach metamorphic competence; *B. l. plei* larvae required only 19 days.

Survivorship in the larval cultures was determined at each culture transfer. It was computed as the number of larvae surviving weighted against the number of larvae placed in the culture at hatching. Consequently, the mortality included both the number of larvae found dead and the number that were physically lost during the transfer process. Regression lines were fitted to the data and regression coefficients computed. For each set of data, an

analysis of covariance was performed to determine whether cultures of different egg masses differed significantly within the same species.

The survival of two different series of cultures of *Aplysia californica* is shown in Figure 3. Line A represents a second generation of larvae (n = 1846) raised through metamorphosis in the laboratory. Line B represents larvae (n = 1516) produced by a wild adult collected at Cedar Key, Florida. These data were significantly different (α = 0.01). In each case, a high proportion of larvae (55–80%) survived to competency from days 34 to 44 and, of those exposed to substrates, 80–90% metamorphosed in ASW.

The survivorship curves for *Bursatella leachii plei* larvae (Figure 4) represent data from three different egg masses raised in 18 separate cultures (n = 5459). No significant differences (α = 0.01) could be detected between two of the culture sets, so these data were lumped and are shown as regression line A. The third set (line B) was significantly different (α = 0.01) from the other two. A large portion of the initial larval population (50–73%) did survive to competency on days 19 to 29. Of these competent larvae, 70–80% could be induced to metamorphose on appropriate substrates.

Two generations of each species were raised to maturity in the laboratory, using the above techniques. Growth rates of the juveniles varied greatly depending on the size of the individual and availability of macroalgal food. Both species attained sexual maturity within 2 to 3 months after metamorphosis.

DISCUSSION

These techniques for raising aplysiid larvae using artificial seawater produced percent yields as good as or better than techniques previously developed using natural seawater. This success was due, at least in part, to the constancy of the larval environmental conditions and to a reduction in the amount of handling necessary to maintain the cultures. Where daily transfers have been necessary in previously reported procedures (Kriegstein *et al.*, 1974; Switzer-Dunlap & Hadfield, 1977), artificial seawater cultures could be left standing for 5 to 12 days without increases in mortality. The filter apparatus used here allowed rapid transfer of the larvae to new cultures and minimized the stresses of handling and exposure to air. The use of Parafilm as a seal for the culture vessels completely eliminated the problem of larval entrapment at the surface (Franz, 1975) and was easier to use than cetyl alcohol (Switzer-Dunlap & Hadfield, 1977).

The techniques described here were designed for, and can be performed at, any inland laboratory. The restrictions of natural seawater have been eliminated. Although juveniles in this study and others (Switzer-Dunlap & Hadfield, 1981) have been raised to maturity in small aquaria in artificial seawater, at the present time we are tied to coastal supplies of adult food and the larval settlement substrates. Strentii & Blankenship (1978) have had success raising juveniles and adults of *Aplysia brasiliana* on commercially available dried seaweed. Capo *et al.* (1979) have reported the successful culture in the laboratory of the red alga *Agardhiella subulata* (C. Agardh) Kraft & Wynne (1979) that supports the metamorphosis and postlarval development of *Aplysia californica* Cooper, 1863. More work is needed on the isolation and characterization of the metamorphic inducer(s) to realize the potential of inland culture.

The use of a defined medium for the culture of aplysiids now affords researchers the opportunity to investigate the chemical requirements of larval development and the effects of changing environments on adult neurophysiology and behavior.

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